

Vaccinated C57BL/6 Mice Develop Protective and Memory T Cell Responses to *Coccidioides posadasii* Infection in the Absence of Interleukin-10

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High concentrations of lung tissue-associated interleukin-10 (IL-10), an anti-inflammatory and immunosuppressive cytokine, correlate with susceptibility of mice to *Coccidioides* spp. infection. In this study, we found that macrophages, dendritic cells, neutrophils, and both CD8⁺ and CD4⁺ T cells recruited to *Coccidioides posadasii*-infected lungs of nonvaccinated and vaccinated mice contributed to the production of IL-10. The major IL-10-producing leukocytes were CD8⁺ T cells, neutrophils, and macrophages in lungs of nonvaccinated mice, while both Foxp3⁺ and Foxp3⁻ subsets of IL-10⁺ CD4⁺ T cells were significantly elevated in vaccinated mice. Profiles of the recruited leukocytes in lungs revealed that only CD4⁺ T cells were significantly increased in *IL-10*^{-/-} knockout mice compared to their wild-type counterparts. Furthermore, *ex vivo* recall assays showed that CD4⁺ T cells isolated from vaccinated *IL-10*^{-/-} mice compared to vaccinated wild-type mice produced significantly higher amounts of IL-2, gamma interferon (IFN- γ), IL-4, IL-6, and IL-17A in the presence of a coccidioidal antigen, indicating that IL-10 suppresses Th1, Th2, and Th17 immunity to *Coccidioides* infection. Analysis of absolute numbers of CD44⁺ CD62L⁻ CD4⁺ T effector memory T cells (T_{EM}) and IFN- γ - and IL-17A-producing CD4⁺ T cells in the lungs of *Coccidioides*-infected mice correlated with better fungal clearance in nonvaccinated *IL-10*^{-/-} mice than in nonvaccinated wild-type mice. Our results suggest that IL-10 suppresses CD4⁺ T-cell immunity in nonvaccinated mice during *Coccidioides* infection but does not impede the development of a memory response nor exacerbate immunopathology of vaccinated mice over at least a 4-month period after the last immunization.

Coccidioides is a fungal pathogen and the causative agent of a human respiratory disease known as coccidioidomycosis, or San Joaquin Valley fever (1). Human infections typically occur by inhalation of spores (arthroconidia) released into the air from the saprobic phase of the soilborne fungus. In the approximately 40% of human exposures that result in symptomatic infection, the initial clinical manifestation is characterized by onset of an acute respiratory response that can occur within 1 to 3 weeks after inhalation of the pathogen. In a few patients (<5%), *Coccidioides* species infections may progress to life-threatening, chronic pneumonia, extrapulmonary nonmeningeal disease, or meningitis. The latter is the most feared complication of coccidioidomycosis (1). The number of reported cases of primary coccidioidal pneumonia in Arizona and California has significantly increased during the last decade (2), which has raised the level of awareness among people who live in regions where this mycosis is endemic. Development of a vaccine and effective therapeutic strategies against coccidioidomycosis would promote the well-being of at-risk populations in the areas of endemicity.

Interleukin-10 (IL-10) is a pleiotropic cytokine with anti-inflammatory and immunosuppressive functions and the ability to impact both innate and adaptive immunity to microbial infections (3–5). Studies using IL-10 knockout mice have suggested that this cytokine is an essential immune regulator in a variety of fungal infections, including infections caused by *Candida* spp. (6), *Cryptococcus neoformans* (7), *Histoplasma capsulatum* (8), *Pneumocystis carinii* (9), and *Aspergillus fumigatus* (10). A correlation has been revealed between susceptibility to *Coccidioides* infection and the amount of IL-10 produced (11–14). Loss of IL-10 production significantly improves the outcome of coccidioidomycosis in nonvaccinated mice (12, 13). IL-10 can exert direct inhibition on CD4⁺ T cell proliferation and cytokine synthesis (15). In the latter

case, IL-10 has been shown to suppress the production of IL-2, gamma interferon (IFN- γ), IL-4, and IL-5 (16) and, thereby, hamper protective responses of both Th1 and Th2 cells during early stages of microbial and viral infections (15, 17). Recently, IL-10 has also been shown to inhibit murine macrophages and T cells in the secretion of Th17-related cytokines (18). The latter are required for development of Th17-type immunity, which is essential for vaccine-induced protection against *Coccidioides* infection and other dimorphic fungal diseases (19, 20). Thus, treatment with anti-IL-10 antibody and vaccination strategies aimed at neutralizing excess IL-10 following microbial infection should provide therapeutic advantages (21, 22).

On the other hand, IL-10 is required to control fungal infections caused by *Candida albicans* and *P. carinii* (9, 23) as well as numerous viral, bacterial, and parasitic pathogens (24–26). Although IL-10-deficient mice infected with *Candida* revealed significantly reduced fungal burden, the mice presented with severe inflammatory pathology and susceptibility to reinfection (23). An attempt to treat *Leishmania major* infection in mice by immunization with an IL-10 peptide-based vaccine revealed increased parasitic burden and exacerbated disease (27). These contradictory effects of IL-10 raise concerns about application of supple-

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mental IL-10 therapy to treat inflammatory diseases or neutralization of IL-10 to improve the efficacy of vaccines against microbial infections (21, 22).

Despite the considerable information available regarding the regulatory functions of IL-10 for the immune response and in immunopathology, there is less known about the major sources of this cytokine during specific microbial infections. IL-10 can be produced by CD4⁺ T regulatory (Treg) cells, CD8⁺ T cells, and numerous members of the innate immune repertoire, including dendritic cells (DCs), macrophages, mast cells, natural killer cells, neutrophils (polymorphonuclear leukocytes [PMNs]), and B cells (3). In a murine model of acute brucellosis, CD4⁺ CD25⁺ T cells were identified as the major source of IL-10 (28). These cells were shown to play an important role in modulating the early immune response to *Brucella abortus* infection. Similarly, T-cell-derived, but not B-cell-derived, IL-10 was reported to contribute to the suppression of the antigen-specific CD4⁺ T-cell response to a helminth parasite infection in mice (29). In the case of *Yersinia enterocolitica* infection, the main sources of IL-10 were neutrophils (30). In this study, we explored the following questions related to the IL-10 response to *Coccidioides posadasii* infection. (i) What are the cellular sources of IL-10 in vaccinated versus nonvaccinated C57BL/6 mice following pulmonary infection? (ii) Are the composition and numbers of immune cells in the lungs of *Coccidioides*-infected mice altered in the absence of IL-10? (iii) Can IL-10-deficient mice develop long-term T-cell immunity and polarization of the CD4⁺ T-cell response to infection? (iv) Does ablation of IL-10 cause immunopathology during early stages of coccidioidomycosis?

MATERIALS AND METHODS

Fungal strain, growth conditions, and spore preparation. The virulent fungal strain used to challenge mice in this study was clinical isolate C735 of *Coccidioides posadasii*. A previously described genetically engineered, live, attenuated mutant strain (Δ cts2/ Δ rd1/ Δ cts3) derived from the parental C735 isolate was employed as a vaccine and designated Δ T (19, 31). The saprobic phases of both the wild-type (WT) and mutant strains were grown on GYE agar (1% glucose, 0.5% yeast extract, 1.5% agar) for 3 to 4 weeks at 30°C to generate a confluent layer of spores on the surface of the solid culture medium. Spore suspensions used for vaccination or intranasal challenge of mice as described below were prepared as previously reported (32). All culturing and preparatory procedures that involved live cells of *C. posadasii* were conducted in a biosafety level 3 (BSL3) laboratory.

Mouse strain. Breeder pairs of C57BL/6 and B6.129P2-IL10^{tm1Cgn/J} (IL-10^{-/-}) mice were purchased from Jackson Laboratory. Mice were housed in a specific-pathogen-free (SPF) animal facility at the University of Texas at San Antonio (UTSA) and handled according to guidelines approved by the university's Institutional Animal Care and Use Committee. Both strains of mice were gender matched. Animals 8 to 10 weeks old with an average weight of 18 to 22 g were used in this study. Mice were relocated to an animal biosafety level 3 laboratory prior to vaccination and infection.

Vaccination protocol, animal challenge, and evaluation of protection. Primary immunization of C57BL/6 and IL-10^{-/-} mice with 5.0 × 10⁴ viable spores of the live Δ T vaccine in 100 μ l phosphate-buffered saline (PBS) was performed by the subcutaneous route in the abdominal region. This initial vaccination step was followed 14 days later with a boost of 2.5 × 10⁴ spores by the same route of immunization. Control mice were injected with PBS following the same vaccination protocol as above. The animals were challenged 16 weeks after completion of the vaccination protocol by intranasal instillation with a suspension of approximately 80 viable spores of the virulent, parental isolate of *C. posadasii* (C735) in 35

μ l PBS as previously reported (31). The fungal burden in the lungs and spleen was determined at the time when the animal approached the moribund state or at 14 days postchallenge (DPC) by plating serial dilutions of separate lung and spleen homogenates on GYE agar containing 50 μ g/ml chloramphenicol, as reported elsewhere (31). The number of CFU of *Coccidioides* was expressed on a log scale and reported for each group of 10 animals as previously described (31). Survival studies of vaccinated versus nonvaccinated mice were conducted over 60 days postchallenge as reported previously (31).

Cytokine assays. Concentrations of IL-10 in supernatants of lung homogenates were compared between nonvaccinated and Δ T-vaccinated mice sacrificed at 5, 7, 9, 11, and 14 DPC (4 animals per group). Lung homogenates of individual mice were prepared as previously described (33), supernatants were obtained by centrifugation (8,000 × g at 4°C for 10 min), and samples were stored at -80°C until ready for analysis. Assays of cytokine and chemokine concentrations were conducted using a Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA) as previously reported (33).

Quantitative RT-PCR assays. Total RNA was extracted from isolated pulmonary cells of *Coccidioides*-infected lungs of nonvaccinated and vaccinated C57BL/6 mice (three animals per group) using an RNeasy kit (Qiagen, Valencia, CA). The samples were treated with DNase (Promega, San Luis Obispo, CA) to remove traces of contaminating DNA. Reverse transcription (RT) was performed using 5 μ g total RNA in a 50- μ l reaction mixture containing oligo(dT) and SuperScript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The relative amounts of specific gene transcripts encoding IL-10 and the forkhead box P3 transcription factor (*Foxp3*) (34) in the lungs of nonvaccinated and vaccinated mice were examined by quantitative RT-PCR (QRT-PCR) as previously reported (32). The data are presented as the mean fold change \pm the standard error of the mean (SEM).

Quantification of Treg cells. Regulatory T cells are characterized by expression of CD3, CD4, and CD25 surface markers as well as expression of the *Foxp3* transcription factor gene. Total pulmonary leukocytes were isolated from nonvaccinated mice or mice immunized with the Δ T vaccine and sacrificed at 5 to 14 days postchallenge (3 mice per group) as previously reported (19). A standard methodology was employed for direct monoclonal antibody (MAb) labeling and enumeration of selected pulmonary leukocyte phenotypes by fluorescence-activated cell sorting (FACS) with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) as previously described (19). Isolated pulmonary leukocytes were blocked and labeled with fluorochrome-conjugated monoclonal antibodies against CD3 (clone 17A2), CD4 (clone RM4-5), and CD25 (clone PC61). The cells were then chemically fixed and permeabilized using a mouse Foxp3 buffer set (BD Bioscience) and incubated with 0.25 μ g of fluorochrome-conjugated anti-Foxp3 MAb (clone MF23) in 40 μ l of FACS buffer for 30 min at 4°C. The absolute number of Treg cells was determined by multiplying the percentage of the gated population by the total number of CD45⁺ cells.

Characterization of IL-10-producing leukocyte subpopulations by flow cytometry. Pulmonary leukocytes were isolated from nonvaccinated mice or mice vaccinated with the Δ T vaccine as well as naive (untreated) IL-10^{-/-} mice. The latter were used as negative controls for intracellular staining of IL-10. Pulmonary cells were stimulated with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, St. Louis, MO) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop and GolgiPlug (BD Biosciences) in 10% fetal bovine serum (FBS)-supplemented RPMI 1640 for 5 h at 37°C. After stimulation, aliquots of cells were first stained for surface markers of T cells (CD4, CD8 α , and CD45) and B cells (CD19 and CD45) in separate wells and then stained for intracellular IL-10 as previously described (19). A separate aliquot was first labeled with monoclonal antibodies against surface markers CD3 and CD4, followed by intracellular labeling for Foxp3 and IL-10 to determine numbers of IL-10-producing Treg cells. Pulmonary cells were gated on CD45⁺ leukocytes for T- and B-cell panels and then analyzed for IL-10 labeling. The amount

of baseline label of IL-10 in leukocytes (CD45⁺) isolated from IL-10^{-/-} mice was used as the control. Percentages of CD4⁺ CD8⁻, CD4⁻ CD8⁺, and CD19⁺ cells in the CD45⁺ IL-10⁺ population were determined and used to calculate the absolute number of IL-10-producing CD4⁺ T cells, CD8⁺ T cells, and B cells, respectively. The double-positive CD4⁺ CD8⁺ cells, which could also be DCs, were not included within the gates. Pulmonary leukocytes identified as IL-10⁺/CD8⁻ CD4⁺ Foxp3⁺ were defined as pulmonary Treg cells. Aliquots of IL-10⁺-labeled, PMA/ionomycin-activated pulmonary cells were stained for surface markers to identify different types of granulocytes (CD11b, Ly6G, and CD11c). FACS data were first gated on cells which were IL-10⁺ and then further analyzed for their expression of Ly6G, CD11b, and CD11c. Percentages of Ly6G⁺ CD11b⁺, CD11b⁺ CD11c⁺, and CD11b⁺ CD11c⁻ Ly6G⁻ cells in the IL-10⁺ population were determined and used to calculate the absolute number of IL-10-producing neutrophils, dendritic cells, and macrophages, respectively.

Quantification of leukocyte cell types in *Coccidioides*-infected lungs. Flow cytometry was used to determine the numbers of CD4⁺ T cells, CD8⁺ T cells, B cells, natural killer cells (NK), neutrophils (PMNs), alveolar macrophages (AM), inflammatory macrophages (IM), and DCs in the lung homogenates as previously described (19, 33). The gating strategies were the following: CD3⁺ CD4⁺ CD8⁻, CD3⁺ CD4⁻ CD8⁺, CD19⁺, CD161c (NK1.1)⁺ CD3⁻, Ly6G⁺ CD11b⁺ CD11c⁻, intermediate levels of CD11b (CD11b^M) CD11c⁺, Mac3⁺ CD11c⁻ Ly6G⁻, and high levels of CD11b (CD11b^H) CD11c⁺, respectively (19, 32, 33).

Recall assays of CD4⁺ T cells. Splenocytes of wild-type C57BL/6 and IL-10^{-/-} mice immunized with the ΔT vaccine were separately harvested, pooled, and macerated for isolation of CD4⁺ T cells by using a CD4⁺ T-cell isolation kit (Miltenyi Biotec, Inc., Gladbach, Germany) as previously reported (35). The purity of CD4⁺ T cells was verified by staining the cells with fluorescein-conjugated anti-CD3 and anti-CD4 MAbs, followed by flow cytometry. The purity of CD4⁺ T cells was routinely above 90%. The isolated CD4⁺ T cells were cultured in 24-well plates (2.5 × 10⁶ cells/well) with 2.5 × 10⁶ splenocytes isolated from naive IL-10^{-/-} mice in 2 ml RPMI 1640 containing 10% heat-inactivated FBS, 1% streptomycin (Strep), and 1% L-glutamine, to which 40 μg/ml of a coccidioidal antigen extract (T27K) was added. Examination of *in vitro* T-cell stimulation was conducted by incubation of immune splenocytes in growth medium alone or with the addition of T27K antigen (19) for 72 h at 37°C in the presence of 5% CO₂. T cells incubated in growth medium alone served as negative controls. After incubation, a cocktail of protease inhibitors (Complete, EDTA-free; Roche Diagnostics, Pleasanton, CA) was added to each well, and culture supernatants were collected from the centrifuged samples (11,950 × g for 10 min at 4°C). The concentrations of selected cytokines were determined by using a Bio-Plex suspension array system.

Intracellular cytokine staining. Aliquots of pulmonary leukocytes were stimulated with anti-CD3 and anti-CD28 in the presence of GolgiStop in 10% FBS-supplemented RPMI 1640 for 4 h at 37°C. Permeabilized cells were stained with selected fluorochrome-conjugated monoclonal antibodies (anti-IFN-γ, anti-IL-5, or anti-IL-17A) to determine absolute numbers of the specific cytokine-producing CD4⁺ T cells as previously described (19, 36). The leukocytes were gated for CD4⁺ CD8⁻ T cells, and numbers of cytokine-producing cells were determined. The absolute numbers of the specific cytokine-producing CD4⁺ T cells relative to the total lung-infiltrating leukocytes per lung homogenate at 8 and 12 days postchallenge were calculated by multiplying the percentage of each gated population by the total number of viable pulmonary leukocytes determined by hemocytometer counts as described above.

Histopathology. Comparative histopathology was conducted with excised whole lung organs from *Coccidioides*-infected, nonvaccinated, and vaccinated wild-type C57BL/6 and IL-10^{-/-} mice. The animals were sacrificed at 14 days postchallenge (4 mice per group) as reported elsewhere (31). Tissue fixation and embedding procedures were performed as described previously (31). Paraffin sections (5 μm thick) were stained with hematoxylin and eosin (H&E) or Gomori methenamine silver stain

(GMS) by standard procedures. Sections were examined using a Leica DMI6000 microscope equipped with an automated turboscan stage (Objective Imaging, Ltd., Cambridge, United Kingdom), and microscope images of infected lung tissue were acquired and analyzed using Surveyor software (Objective Imaging) as reported elsewhere (32).

Statistical analyses. The Student Newman-Keuls test, a type of analysis of variance statistical method for multisample comparisons, was used to analyze the enzyme-linked immunosorbent assay results, cytokine concentrations, absolute numbers of lung-infiltrating immune cells, and percentages of specific cytokine-producing T cells as previously reported (36). All pairwise comparisons of the fungal burden, measured as the CFU between nonvaccinated versus vaccinated mice and between wild-type versus IL-10^{-/-} mice, were analyzed using the Mann-Whitney U test as reported previously (31). Survival data were examined by the Kaplan-Meier test using log rank analysis to compare survival plots, as reported previously (31). A *P* value of <0.05 was considered statistically significant.

RESULTS

Vaccinated C57BL/6 mice revealed early but limited production of IL-10 after *Coccidioides* infection. C57BL/6 mice were vaccinated with the live, attenuated vaccine strain (ΔT) and challenged with a potentially lethal dose of *Coccidioides* spores as previously described (19, 31, 32), except that the interval between the last vaccination and challenge was extended from 4 to 16 weeks. At 8 weeks postvaccination, there was no detectable live, attenuated *Coccidioides* ΔT cells in any of the tissue or organ homogenates examined, including cutaneous tissue at the injection site, draining lymph nodes, lungs, spleen, liver, and kidneys (the detection limit was 5 CFU per organ). We extended the interval prior to challenge to 16 weeks in order to evaluate the memory T-cell response. Cytokine assays of infected lung homogenates of mice revealed distinct differences in the kinetics of IL-10 production between the vaccinated (ΔT) and nonvaccinated mice during the first 14 DPC (Fig. 1A). The concentration of IL-10 was significantly elevated in vaccinated mice starting at 7 DPC, peaked at 9 DPC, and then declined between 11 and 14 DPC (Fig. 1A). In contrast, lung homogenates of nonvaccinated mice showed a steady increase in IL-10 production between 7 and 14 DPC, which correlated with the increase in fungal burden in the lungs and dissemination of the pathogen to the spleen, as previously reported (19). Since recruitment of leukocyte subpopulations in lungs of WT mice has been shown to be comparable at 12 and 14 DPC (19), we decided to conduct our immunological assays at 8 and 12 DPC, which represented the time points before and after dissemination occurred. In addition, because IL-10 gene expression is expected to precede an increase in IL-10 cytokine production, QRT-PCR analysis of the amounts of IL-10 transcript was conducted at these same time points before the peaks of IL-10 concentration were detected in the lungs of vaccinated mice (9 DPC) and nonvaccinated mice (14 DPC). The results were coincident with upregulation of IL-10 production in the lungs of nonvaccinated but not vaccinated mice (Fig. 1B). The amount of IL-10 transcript in the lungs of vaccinated mice showed no significant change between 8 and 12 DPC, while upregulation of IL-10 expression in lung homogenates of nonvaccinated mice revealed an approximate 35-fold increase during this same period postchallenge. These observations support previous findings indicating that there is a direct correlation between amounts of IL-10 production and susceptibility to *Coccidioides* infection (12, 13).

Treg cells contributed to IL-10 production in the vaccinated mice. We next attempted to determine which lung-infiltrated im-

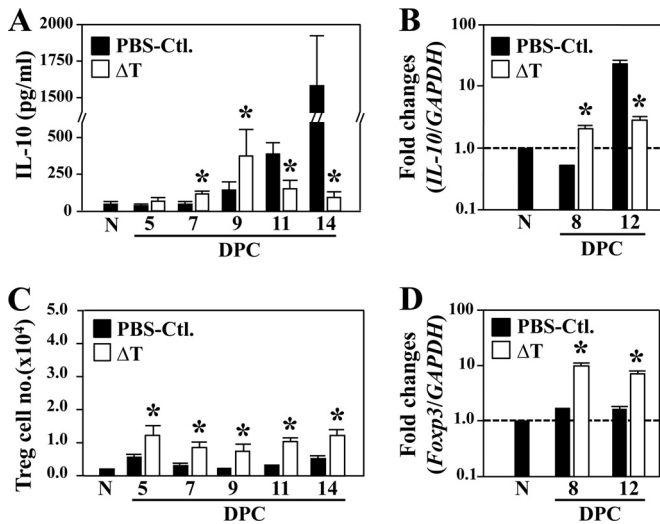


FIG 1 IL-10 production in lungs of vaccinated (ΔT) and nonvaccinated (PBS-Ctl.) C57BL/6 mice did not directly correlate with recruitment of $CD4^+$ Treg cells. (A and B) IL-10 cytokine levels (A) and fold changes of transcripts (B) in lung homogenates at the indicated DPC following intranasal challenge with approximately 60 to 80 *Coccidioides* spores were determined for C57BL/6 mice that were challenged at 16 weeks after the final vaccination. Nonvaccinated and noninfected mice (N) of the same age and gender were included for determination of the baseline amounts of IL-10 transcript and protein. (C and D) The absolute number of $Foxp3^+ CD4^+ CD25^+$ Treg cells (C) in lung homogenates of the mice was determined at the indicated time points. Total RNA was also isolated from duplicated sets of mice and used for determination of the fold changes of *Foxp3* expression at 8 and 12 DPC (D). The assays were conducted in two independent experiments. Data are means \pm SEM of 4 mice per group. Asterisks indicate $P < 0.05$ compared to nonvaccinated mice.

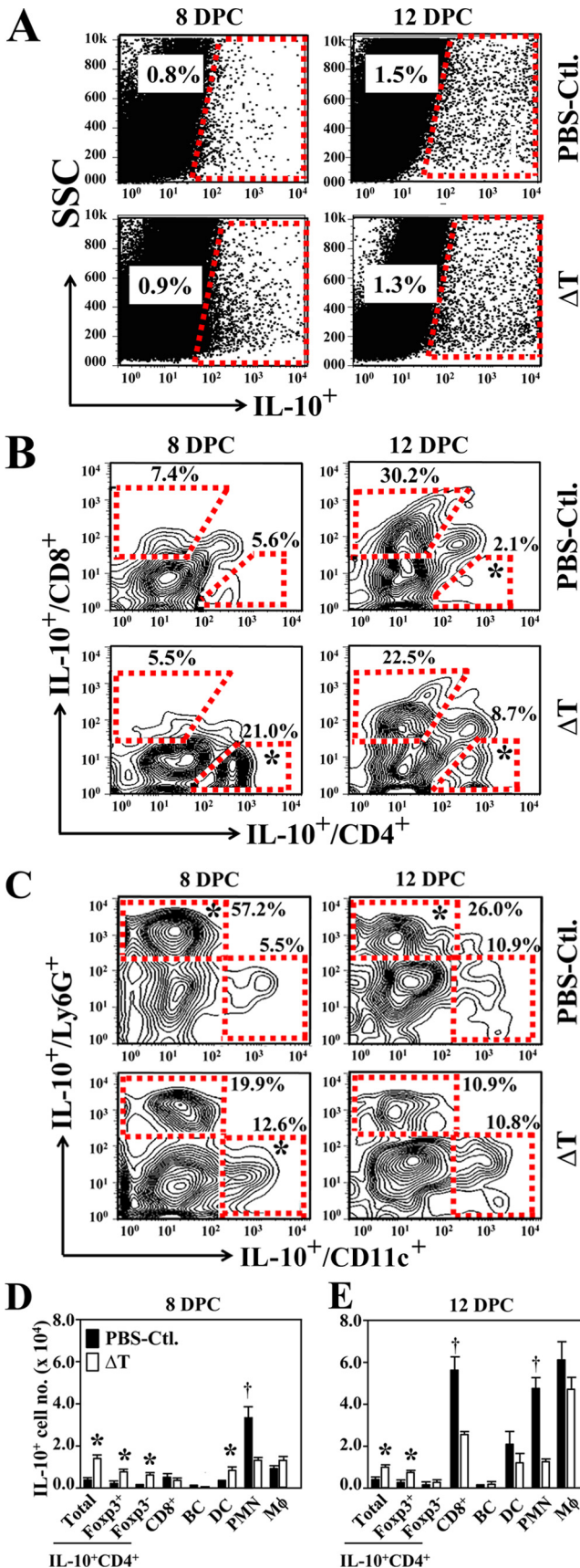
immune cells contributed to IL-10 production in the *Coccidioides*-infected lungs. Since it is well established that $CD3^+ CD4^+ Foxp3^+$ T cells (Treg phenotype) can produce high amounts of IL-10 during microbial infection (4, 5), we employed cytometric techniques to determine the absolute numbers of Tregs that were recruited to the *Coccidioides*-infected lungs of vaccinated and nonvaccinated mice during the initial 14 days postchallenge. Our results revealed that Treg cells were sustained at a significantly elevated number in vaccinated compared to nonvaccinated mice during this period (Fig. 1C). We also compared expression of *Foxp3* in lung homogenates of the two groups of infected mice (Fig. 1D). The amount of *Foxp3* transcript was significantly higher in homogenates of vaccinated than in nonvaccinated mice at 8 and 12 DPC, and both showed little change during this interval. Unexpectedly, the kinetics of Treg recruitment did not correlate with the peak of IL-10 production in nonvaccinated mice, suggesting that other immune cells in lung homogenates of this group of mice contribute to the sharp increase in IL-10 concentration.

Comparison of the relative contributions of IL-10 production by immune cell subpopulations recruited to lungs of vaccinated versus nonvaccinated mice. We systematically investigated leukocyte subpopulations that were capable of producing IL-10 in *Coccidioides*-infected lungs. The percentages of leukocytes positive for IL-10 production in lungs of vaccinated mice were comparable to nonvaccinated mice at 8 and 12 DPC (Fig. 2A). Further analyses showed that a significantly higher percentage and number of the gated IL-10-producing lymphocytes were $CD4^+$ T cells of vaccinated than in nonvaccinated mice at 8 and 12 DPC

(Fig. 2B, D and E). The total numbers of $CD4^+ IL-10^+$ T cells were further examined for their expression of *Foxp3*. The numbers of IL-10-producing $Foxp3^+ CD4^+$ cells were significantly elevated in the lungs of vaccinated mice at 8 and 12 DPC (Fig. 2D and E). On the other hand, the lungs of nonvaccinated mice had a higher percentage and number of $CD8^+$ T cells producing IL-10 at 12 DPC (Fig. 2B and E). Interestingly, a significantly elevated percentage and total number of PMNs in *Coccidioides*-infected lungs of nonvaccinated mice were capable of producing IL-10 at both 8 and 12 DPC compared to vaccinated mice (Fig. 2C, D, and E). IL-10-producing $CD19^+$ B cells were not detected in *Coccidioides*-infected lungs during the first 2 weeks postchallenge (Fig. 2D and E). IL-10-producing granulocytes, including PMNs, DCs, and macrophages, were the major innate cell types recruited to the *Coccidioides*-infected lungs of both nonvaccinated and vaccinated mice. In summary, our results showed that $CD4^+$ T cells, both $Foxp3^+$ and $Foxp3^-$ cells, and DCs and macrophages were the major activated immune cells that produced IL-10 in the lungs of vaccinated mice, while $CD8^+$ T cells, PMNs, and macrophages were the major cell types that contributed to the sharp increase of IL-10 in the lungs of nonvaccinated mice after 11 days postchallenge (Fig. 1A and 2D and E).

Enhanced recruitment of $CD4^+$ T cells to lungs of $IL-10^{-/-}$ mice during the first 8 days postchallenge. To investigate whether acquisition of leukocyte subpopulations in lungs was altered in the absence of IL-10, we compared the inventory of $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, PMNs, alveolar and inflammatory macrophages, and dendritic cells that had infiltrated *Coccidioides*-infected lungs of WT and $IL-10^{-/-}$ mice at 8 and 12 DPC. The numbers of these examined immune cells of both naive $IL-10^{-/-}$ and WT mice were comparable to vaccinated WT mice before challenge (0 DPC), as we previously reported (19). No significant difference in the numbers of these immune cells was detectable between vaccinated WT and $IL-10^{-/-}$ mice before challenge. Total numbers of $CD45^+$ leukocytes were comparable in lungs of $IL-10^{-/-}$ and wild-type mice during the first 12 DPC. Significantly elevated levels of $CD4^+$ T cells and B cells accompanied by reduced percentages of pulmonary-infiltrating PMNs were observed in lung homogenates of vaccinated compared to nonvaccinated $IL-10$ -deficient mice at 8 and 12 DPC (Fig. 3A to D). In addition, the percentage of $CD8^+$ T cells of vaccinated $IL-10^{-/-}$ mice was significantly higher at 12 DPC than in the nonvaccinated mice (Fig. 3B). These results were comparable to those of the nonvaccinated and vaccinated wild-type mice (Fig. 3A to D) (19), except that a significantly higher percentage of $CD4^+$ T cells was revealed in the lungs of nonvaccinated and vaccinated $IL-10^{-/-}$ mice at 8 DPC (Fig. 3A to D). These results suggested that IL-10 mainly impacts the acquisition of $CD4^+$ T cells following pulmonary infection with *Coccidioides*, particularly during the first 8 days postchallenge.

$CD4^+$ T cells isolated from vaccinated $IL-10^{-/-}$ mice respond *ex vivo* to *Coccidioides* antigen by increased production of Th1-, Th2-, and Th17-type cytokines. We compared cytokine secretion by $CD4^+$ T cells isolated from ΔT -vaccinated wild-type and $IL-10^{-/-}$ mice at 16 weeks after the final step of the immunization protocol. Splenocytes isolated from naive $IL-10^{-/-}$ mice were used as antigen-presenting cells (APCs) to ensure that the source of IL-10 was from $CD4^+$ T cells. The concentrations of selected cytokines, which are indicators of Th1 (Fig. 4A and B), Th2 (Fig. 4C and D), and Th17 (Fig. 4E and F) polarized re-



sponses, were highest in T cells isolated from ΔT-vaccinated *IL-10^{-/-}* mice upon stimulation *ex vivo* with *Coccidioides*-specific T27K antigen ($P < 0.01$). CD4⁺ T cells isolated from nonvaccinated wild-type or IL-10 knockout mice showed minimal *ex vivo* production of the selected cytokines when cocultured with the T27K antigen. Likewise, isolated immune CD4⁺ T cells cultured in the absence of T27K consistently showed low levels of cytokine production. Taken together, these data suggest that vaccine-induced CD4⁺ memory T cells differentiated in both wild-type and *IL-10^{-/-}* mice at least by 16 weeks after the final vaccination step. On the other hand, the presence of IL-10 suppressed the secretion of cytokines by CD4⁺ T cells in a self-regulatory manner.

Vaccinated *IL-10^{-/-}* mice undergo expansion of effector memory T cells during the first 8 DPC. To determine whether the elevated, *ex vivo* response of CD4⁺ T cells isolated from vaccinated *IL-10^{-/-}* mice described above translated into changes in T effector cell memory (T_{EM}) function in the infected sites, we isolated pulmonary CD4⁺ T cells from each mouse group and compared their expression of CD44 and CD62L at 8 and 12 DPC. Both vaccinated and nonvaccinated *IL-10^{-/-}* mice consistently showed higher percentages and numbers of CD44⁺ CD62L⁻ CD4⁺ T cells (T_{EM}) during the initial 12 days postchallenge compared to wild-type mice (Fig. 5A). Phenotypic analysis of activated, pulmonary CD4⁺ T cells revealed that significantly higher numbers of Th1 (IFN-γ⁺) and Th17 (IL-17A⁺) cells were present in lungs of vaccinated and nonvaccinated *IL-10^{-/-}* mice than WT mice at 8 DPC (Fig. 5B). At 12 DPC, significant differences were observed between absolute numbers of Th1 and Th17 cells isolated from nonvaccinated *IL-10^{-/-}* versus WT mice, while vaccinated mice of both strains had comparable numbers of Th1 and Th17 cells. No significant difference was detected in the absolute numbers of Th2 (IL-5) cells between these two groups of mice during the first 12 days postchallenge (Fig. 5B).

Nonvaccinated *IL-10^{-/-}* mice showed significantly better clearance of *Coccidioides* and prolonged survival compared to nonvaccinated WT mice. Relative degrees of protection against coccidioidomycosis were determined by measuring numbers of CFU in the lungs and spleen of wild-type and *IL-10^{-/-}* mice at 14 DPC. The mean CFU in the lungs of nonvaccinated *IL-10^{-/-}* mice ($5.2 \pm 1.4 \log_{10}$) was significantly lower than that of wild-type mice ($6.9 \pm 1.3 \log_{10}$; $P = 0.0002$) (Fig. 6A). Similarly, nonvaccinated *IL-10^{-/-}* mice showed significantly reduced fungal burdens in their spleens ($2.6 \pm 1.7 \log_{10}$) than did nonvaccinated WT mice

FIG 2 Diverse subsets of lymphocytes and granulocytes produced IL-10 in the lungs during *Coccidioides* infection. Pulmonary cells were isolated from the nonvaccinated (PBS-Ctl.) or vaccinated (ΔT) C57BL/6 mice at 8 or 12 DPC. (A) Dot plots of gated IL-10-producing cells in the pulmonary leukocyte population (CD45⁺). (B) Percentages of CD4⁺ versus CD8⁺ cells in the gated IL-10⁺ populations. Numbers juxtaposed to the gates give the mean percentage of the subset populations within the gated IL-10⁺ cells. (C) Percentages of CD11c⁺ versus Ly6G⁺ subsets of granulocytes, which represented DCs and PMNs in the gated IL-10⁺ cell population. (D and E) Total numbers of IL-10-producing CD4⁺ T cells in the lungs were further divided into Foxp3⁺ and Foxp3⁻ subpopulations. The numbers of IL-10-producing CD8⁺ T cells, B cells (BC), dendritic cells, neutrophils (PMN), and macrophages (Mφ) per lung organ were also measured as described in Materials and Methods at 8 and 12 DPC. Asterisks indicate significantly higher numbers of IL-10-producing cell numbers in vaccinated compared to nonvaccinated mice, while daggers indicate higher numbers in the nonvaccinated mice compared to vaccinated mice ($P < 0.05$). Data are representative of 2 independent experiments with similar results.

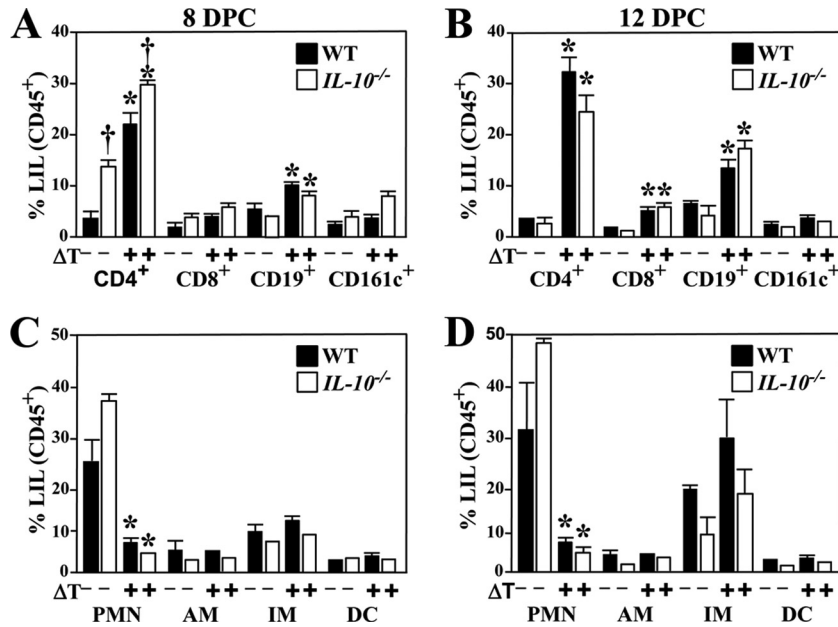


FIG 3 Enhanced acquisition of CD4⁺ T cells in lung homogenates of *Coccidioides*-infected *IL-10*^{-/-} mice. Pulmonary cells were isolated from the nonvaccinated or vaccinated *Coccidioides*-infected WT and *IL-10*^{-/-} mice that were sacrificed at 8 DPC (A and C) or 12 DPC (B and D). Percentages of each immune cell subset within the gated CD4⁺ population were determined as described in Materials and Methods. Data are presented as means ± SEM of 4 mice per group. Asterisks indicate significant differences between the vaccinated and nonvaccinated mice of the same strain, while the dagger represents a significant difference between the *IL-10*^{-/-} and WT mice ($P < 0.05$).

($4.1 \pm 1.8 \log_{10}$; $P = 0.0037$) (Fig. 6B). As expected, both vaccinated *IL-10*^{-/-} and wild-type mice showed comparable reductions of fungal burden in both their lungs ($2.2 \pm 1.1 \log_{10}$ and $2.3 \pm 0.9 \log_{10}$, respectively) and spleens ($1.1 \pm 0.4 \log_{10}$ and $1.6 \pm 0.8 \log_{10}$, respectively). Additional comparative studies revealed that the nonvaccinated *IL-10*^{-/-} mice showed a significant increase in the percent survival compared to nonvaccinated wild-

type mice after a lethal intranasal challenge with approximately 80 spores ($P < 0.01$) (Fig. 6C). Both vaccinated *IL-10*^{-/-} and wild-type mice showed 100% survival after 60 days postchallenge ($P < 0.001$) (Fig. 6C).

Comparative histopathology of nonvaccinated *IL-10*^{-/-} and wild-type mice showed differences in concentrations of parasitic cells in infected lung tissue. At 14 DPC, lungs of both non-

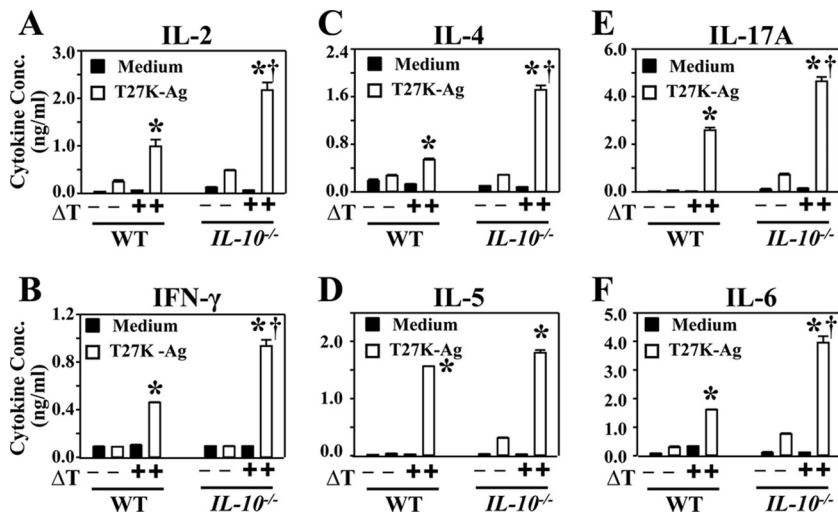


FIG 4 CD4⁺ T cells isolated from vaccinated *IL-10*^{-/-} mice secreted higher amounts of Th1-, Th2-, and Th17-type cytokines upon stimulation with *Coccidioides* antigen than did nonvaccinated mice. CD4⁺ T cells were isolated from splenocytes of WT and *IL-10*^{-/-} mice immunized with the ΔT vaccine or PBS at 16 weeks postvaccination. Isolated CD4⁺ T cells were cultured in the presence or absence of *Coccidioides* T27K antigen. Concentrations of representative Th1 cytokines (IL-2 and IFN-γ) (A and B), Th2 cytokines (IL-4 and IL-5) (C and D), and Th17 cytokines (IL-6 and IL-17A) (E and F) in supernatants of antigen-stimulated immune cells were compared. Statistically significant differences in cytokine production between the ΔT-vaccinated and nonvaccinated mice of the same strain are indicated by asterisks ($P < 0.05$). Daggers indicate a significant difference between WT and *IL-10*^{-/-} mice ($P < 0.01$). The results are representative of 2 separate experiments with 4 mice per group.

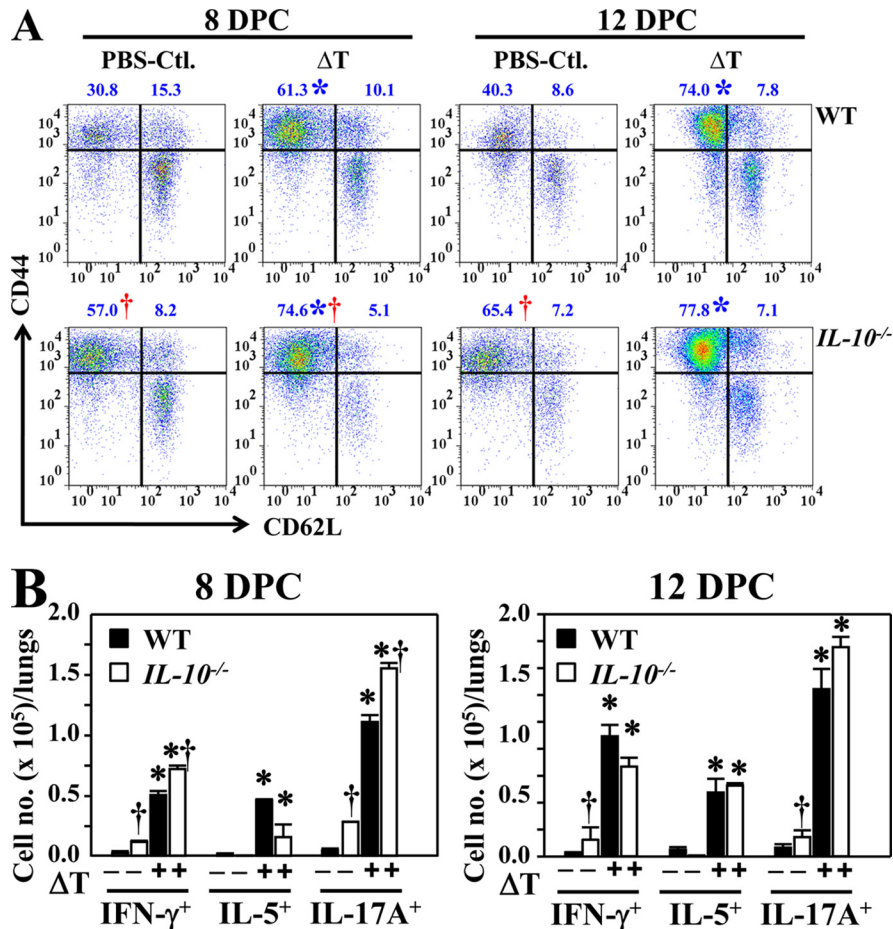


FIG 5 Both ΔT -vaccinated WT and *IL-10*^{-/-} mice revealed elevated percentages and numbers of T_{EM} cells as well as IFN- γ , IL-5, and IL-17A-expressing CD4⁺ T cells compared to nonvaccinated mice. (A) Percentages of T_{EM} (CD4⁺ CD44⁺ CD62⁻) and T_{CM} (CD4⁺ CD44⁺ CD62L⁺) cells within the gated CD4⁺ T-cell population in the lungs of nonvaccinated (PBS-Ctl.) versus vaccinated (ΔT) mice are shown at 8 or 12 DPC. Numbers above the gates indicate the mean percentages of the gated T_{EM} and T_{CM} subsets. (B) The cell numbers of gated, specific cytokine-producing CD4⁺ T cells per lung were determined by intracellular cytokine staining. Asterisks indicate significantly higher cell numbers of responsive T-cell subpopulations in lungs of vaccinated compared to nonvaccinated mice, while the daggers indicate higher numbers of selected cytokine-producing cells in *IL-10*^{-/-} mice compared to WT counterparts. Error bars indicate standard errors. The results reported are representative of two independent experiments.

vaccinated WT and *IL-10*^{-/-} mice had coalescing abscesses that constituted over 50% of the organs. While 50% of the vaccinated WT mice had 1 to 2 small granulomas (~1 to 2 mm in diameter), the others revealed no gross evidence of abscess in their lungs. No vaccinated *IL-10*^{-/-} mice had visible lesions. Cross-sections of visible abscesses in lungs of both nonvaccinated WT and *IL-10*^{-/-} mice were examined (4 mice per strain). Since most of the vaccinated mice did not show any visible lesions in their lungs, we randomly examined tissue sections obtained from anterior, middle, and posterior regions (6 sections per mouse). Our results revealed that both nonvaccinated WT and *IL-10*^{-/-} mice had intense suppurative inflammation associated with *Coccidioides* infection (Fig. 7A to D), although there were significant differences between the CFU in the lungs of these two strains of mice (see Fig. 6A). Sectioned lungs of wild-type mice revealed large numbers of spherules in various stages of development. When viewed at higher magnification (Fig. 7B), densely stained regions corresponding to concentrated inflammatory cells were visible, juxtaposed to mature spherules. Most of the spherules had ruptured and released their endospores. Inflammatory cells appeared to

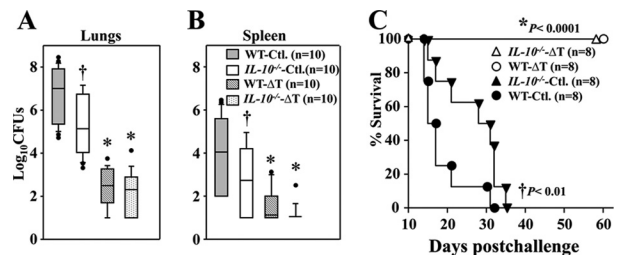


FIG 6 Both vaccinated WT and *IL-10*^{-/-} mice significantly reduced the fungal burden in their lungs and spleen and showed prolonged survival compared to nonvaccinated mice. The numbers of CFU of *C. posadasii* detected in dilution plate cultures of lung (A) and spleen (B) homogenates of WT and *IL-10*^{-/-} mice that had been vaccinated with the ΔT vaccine (ΔT) or immunized with PBS (Ctl.) as controls are reported at 14 DPC. All mice were challenged by the intranasal route with 80 viable spores isolated from the virulent strain. The horizontal lines indicate the mean CFU determinations. (C) Survival plots are presented for WT and *IL-10*^{-/-} mice vaccinated with the ΔT vaccine or treated with PBS as controls. Asterisks indicate a statistically significant difference ($P < 0.05$) between CFU in the lungs and spleen of the vaccinated versus nonvaccinated mice of the same strain, while the daggers indicate significantly reduced CFU in the *IL-10*^{-/-} mice versus the wild-type counterpart. The results are representative of 2 separate experiments.

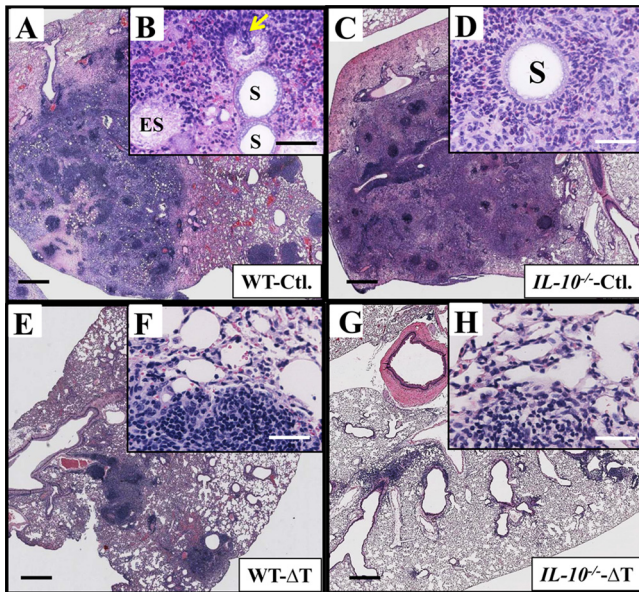


FIG 7 Comparative histopathology of *Coccidioides*-infected lungs revealed greater consolidation of inflammatory tissue in both vaccinated WT and *IL-10*^{-/-} mice compared to nonvaccinated mice. Tissue sections were prepared from lungs of the nonvaccinated (A to D) and vaccinated (E to H) mice at 14 DPC. The large abscesses of the nonvaccinated WT and *IL-10*^{-/-} mice contained concentrated inflammatory cells without apparent organization (A and C). Representative images revealed higher numbers of *Coccidioides* spherules surrounded by areas of necrosis in the nonvaccinated WT mice (B) compared to the *IL-10*^{-/-} mice (D). Inflammatory cells converged to the sites of spherule rupture and in some cases were visible within the spherule matrix of the nonvaccinated mice (arrows in panel B). High numbers of inflammatory cells also surrounded spherules (S) prior to endosporulation in *IL-10*^{-/-} mice. In contrast, both vaccinated wild-type and *IL-10*^{-/-} mice showed localized recruitment of inflammatory cells surrounded by normal tissue (E to H) representative of a protective response, compared to nonvaccinated mice. Bars, 500 μ m (A, C, E, and G) or 100 μ m (B, D, E, F, and H).

have converged to the sites of spherule rupture and in some cases were visible within the spherule matrix (Fig. 7B, arrow). The histopathology of sectioned lungs of nonvaccinated *IL-10*^{-/-} mice revealed significantly fewer spherules. Unlike the wild-type mice, mature spherules that had released their endospores were rarely observed, although dense clusters of infiltrated inflammatory cells were visible, often juxtaposed to young spherules in stages of development prior to endosporulation (Fig. 7D, labeled with an S). The histopathology of lungs obtained from both vaccinated wild-type and *IL-10*^{-/-} mice clearly showed more condensed inflammation and consolidation of the infection than did infected lungs of either nonvaccinated WT or *IL-10*^{-/-} mice (Fig. 7E to H). Rarely were parasitic cells observed within an inflammation region in the lungs of the two groups of vaccinated mice (Fig. 7F and H), and the host tissue adjacent to the abscesses revealed structural features of normal lung tissue.

DISCUSSION

IL-10 has a major impact on the regulation of inflammation during the course of a wide range of infectious diseases (reviewed in references 3 and 5). Extensive evidence supports the notion that persistently elevated IL-10 production during onset of coccidioidomycosis contributes to pathogen proliferation, dissemination, and disease severity (11–14). Fierer and coworkers (13, 14) re-

ported that nonvaccinated, *Coccidioides*-challenged C57BL/6 mice produce higher amounts of IL-10 than resistant DBA2 mice during the early stage of pulmonary infection (10 to 16 days post-challenge) when the pathogen is tissue invasive and disseminates from the lungs to other body organs. To explore the effects of IL-10 on development of vaccine immunity to *Coccidioides* infection, we analyzed the kinetics of IL-10 secretion in the lungs of C57BL/6 mice that had been vaccinated with a protective live, attenuated Δ T vaccine (31). Our results show that in nonvaccinated and vaccinated mice during the onset of pulmonary infection with *Coccidioides*, distinct patterns of IL-10 secretion exist, perhaps involving different sources of the cytokine, and result in unique regulatory pathways. Due to temporal and spatial differences in cell-specific IL-10 expression, it is conceivable that the cytokine has different effects, depending on its source (37). Published studies on the role of IL-10 in coccidioidomycosis have focused on its role in disease progression and impact on innate immunity of nonvaccinated mice. This study is the first to analyze the cellular sources of IL-10 in *Coccidioides*-infected lungs of nonvaccinated and vaccinated C57BL/6 mice and the effects of loss of IL-10 production on vaccine immunity to infection.

We employed *ex vivo* assays and intracellular staining of IL-10 using pulmonary immune cells isolated from the *Coccidioides*-challenged C57BL/6 mice to measure sizes of each subpopulation of immune, cytokine-secreting lymphocytes and granulocytes. Although this approach has limitations of detection of IL-10 production *in situ*, we observed that recruitment of IL-10-producing CD8⁺ T cells, dendritic cells, neutrophils, and macrophages in lungs of nonvaccinated mice were elevated from 8 to 12 DPC, which coincided with the sharp increase of IL-10 concentration during this period. Since recruitment of these leukocyte subpopulations in lungs of nonvaccinated mice was sustained at constant numbers during 12 to 14 DPC, as previously reported (19), it is possible that the same cell types contributed to the sharp increase of IL-10 at 14 DPC (Fig. 1A). In vaccinated and infected mice, CD4⁺ T cells, DCs, and macrophages are the main producers of IL-10. We observed that Treg cells were not elevated in the nonvaccinated mice compared to vaccinated mice. This finding is in accordance with a previous confocal microscopy study that found that an elevated IL-10 concentration was colocalized with T cells and B cells in granulomata of *Coccidioides*-infected human lung tissue, but not with CD4⁺ CD25⁺ Treg cells (38). IL-10-producing CD4⁺, CD8⁺ T cells, and B cells have been reported to regulate immune responses during microbial infection (39–42). However, no IL-10-producing B cells were detected in the lungs of either nonvaccinated or vaccinated mice during the course of *Coccidioides* infection. It has been reported that T-cell-derived, but not B-cell-derived, IL-10 contributes to the suppression of the antigen-specific CD4⁺ T-cell response to helminth parasite infection (29). Similarly, IL-10 produced by T cells but not macrophages or PMNs influences the outcome of *Leishmania* infection (43). To further explore the role of IL-10-producing subsets of immune cells during *Coccidioides* infection, we plan to utilize IL-10 reporter mice that are conditionally deficient in IL-10 production in T cells or CD11b granulocytes (30, 42, 44, 45). These investigations are in progress, and our results will be presented in a separate report.

We observed that lung-infiltrated neutrophils, but not dendritic cells, represented a major elevated myeloid cell type among IL-10-producing pulmonary cells of nonvaccinated mice com-

pared to vaccinated mice. Although *Coccidioides*-infected murine macrophages are capable of producing IL-10 *in vitro* (46), their roles *in vivo* remain to be explored. Neutrophils are one of the first lines of defense against microbial pathogens and are rapidly recruited to *Coccidioides*-infected lungs. Similar results have been reported for systemic infection with *Yersinia enterocolitica*, in which neutrophils are the major source of IL-10 (30). Coactivation of MyD88- and C-type lectin receptor-mediated signal pathways is essential for triggering neutrophils to produce IL-10 during bacterial infections (47). Host recognition of *Coccidioides* is dependent on Toll-like receptor 2 and Dectin-1, a C-type lectin receptor (48). Our preliminary data have shown that both MyD88 and caspase adaptor recruitment domain family member 9 (Card9), an intracellular signal adaptor for C-type lectin receptors, are essential for vaccine immunity to *Coccidioides* infection (unpublished data). Both MyD88- and C-type lectin-mediated pathways appear to be activated during *Coccidioides* infection. Taken together, our data suggest that IL-10-producing CD4⁺ T cells play a regulatory role in the development of protective immunity to *Coccidioides* infection in vaccinated mice, while IL-10-producing CD8⁺ T cells and neutrophils are associated with impairment of resistance to pulmonary coccidioidomycosis in nonvaccinated mice.

Jimenez and coworkers (13) reported that the IL-10 increased susceptibility to pulmonary coccidioidomycosis in mice is partly due to downregulation of inducible nitric oxide synthase 2 (iNOS2) expression. Although iNOS2^{-/-} mice have significantly elevated lung-infiltrating PMNs, macrophages, and dendritic cells at 11 DPC compared to wild-type mice, iNOS2^{-/-} and wild-type mice have comparable fungal burdens (33). It appears that iNOS2 activity has limited influence on the control of *Coccidioides* infection in susceptible mice (33). In our study, we observed that IL-10 mainly impacted acquisition of CD4⁺ T cells to sites of *Coccidioides* infection. Th17 cells have been shown to be the major responding immune cell type during the course of coccidioidomycosis and are essential for protection against *Coccidioides* infection and other dimorphic fungal pathogens (19, 20, 32, 36). Both CD4⁺ Treg and Th17 cells are derived from a common progenitor cell, but they have reciprocal maturation pathways and exert mostly opposing functions (49). We observed that both Treg IL-10-producing CD4⁺ T cells and Th17 cells were elevated in vaccinated mice upon *Coccidioides* infection, possibly as a consequence of maintenance of balanced immune responses through a self-regulatory mechanism of CD4⁺ T cells (4). Both CD4⁺ and CD8⁺ T cells can mediate vaccine immunity and resistance to *Coccidioides* infection (50). Furthermore, CD8⁺ T cells, especially IL-17-producing Th17 cells, can be primed and activated in the absence of CD4⁺ T cells and confer resistance to *Blastomyces* infection (51). The possibility that IL-10 production impedes acquisition of CD8⁺ T cells in lungs of *Coccidioides*-infected mice cannot be excluded.

Although preventing IL-10 production and/or blocking the binding of IL-10 to IL-10 receptors as vaccination strategies may favor the establishment of immune protection, we need to consider the possibility that IL-10 is required for development of long-term memory T-cell immunity (23). IL-10 has been shown to be essential for the normal recruitment of *Aspergillus*-specific CD4⁺ T cells to the airways of infected mice, although the mechanism is not yet known (52). In addition, loss of IL-10 can lead to a detrimental immune response accompanied by both excessive

production of inflammatory cytokines and recruitment of immune cells (26). Memory T cells, which express the activation marker CD44, can be subdivided into two subpopulations: T_{EM} and T central memory (T_{CM}) cells, which are distinguished by their surface expression of the lymph node-homing receptor L-selectin (CD62L). T_{CM} cells have high surface expression of CD62L and reside in the lymphoid tissue, where they replicate and expand, comprising T_{EM} cells. The latter can subsequently be recruited to the infection sites to combat microbial infection (53). T_{EM} cells are the major memory cell type, whereas the size of the CD4⁺ T_{CM} population is transient in *Coccidioides*-infected lungs. As expected, the numbers of T_{EM}, Th1, and Th17 cells were higher in lungs of the nonvaccinated IL-10^{-/-} mice than in nonvaccinated wild-type mice during the first 12 DPC, which is consistent with reduced fungal burden and prolonged survival after *Coccidioides* infection. Both vaccinated IL-10^{-/-} and wild-type mice after infection activate comparable numbers of these three protective cell types and reveal similarly reduced fungal burdens and 100% survival. Durable elevated numbers of antifungal memory CD4⁺ T cells were revealed in both vaccinated and *Coccidioides*-infected IL-10^{-/-} and wild-type mice for at least 16 weeks after vaccination. It is possible that activated T_{EM} cells, including Th1 and Th17 cells, can enhance recruitment of phagocytes to alveoli and promote early reduction of fungal burden while dampening inflammatory pathology at infection sites. Hence, our data show that loss of IL-10 production does not impact the acquisition of T effector memory cells nor exacerbate immunopathology in the lungs of vaccinated mice after pulmonary *Coccidioides* infection. IL-10 depletion has a beneficial influence on protection during the course of coccidioidomycosis in nonvaccinated mice and no alarming effect on vaccine immunity against *Coccidioides* infection.

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